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Anuntalabhochai, S.[1]; Chandej, R.[1]; Phanchaisri, B.[2]; Yu, L.D.[3]; Vilaithong T.[3]; Brown, I.G.[4]

[1)] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

[2)] Institute for Science and Technology Research and Development, P.O. Box 111, Chiang Mai University, Chiang Mai 50202, Thailand

[3)] Fast Neutron Research Facility, Department of Physics, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

[4)] Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720, USA

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Ion-beam-induced deoxyribose nucleic acid transfer

S. Anuntalabhochai and R. Chandej
Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

B. Phanchaisri
Institute for Science and Technology Research and Development, P.O. Box 111, Chiang Mai University, Chiang Mai 50202, Thailand

L. D. Yu and T. Vilaithong[a)]
Fast Neutron Research Facility, Department of Physics, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

I. G. Brown
Lawrence Berkeley National Laboratory, University of California, Berkeley, Berkeley, California 94720

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We report our observations of the interaction of energetic ions with bacterial cells, inducing direct deoxyribose nucleic acid (DNA) transfer into *Escherichia coli (E. coli)*. Argon- and nitrogen-ion beams were used to bombard the bacteria *E. coli* in a vacuum with energy of 26 keV and fluence in the range $0.5-4\times10^{15}$ ions/cm^2. Three DNA plasmids, pGEM2, pGEM-T easy, and pGFP, carrying different marker genes, were subsequently transferred (separately) into the appropriately ion-bombarded bacteria and successfully expressed. The results of this study indicate that ion beams with an energy such that the ion range is approximately equal to the cell envelope thickness, at a certain range of fluence, are able to generate pathways for macromolecule transfer through the envelope without irreversible damage. © *2001 American Institute of Physics.*
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Ion bombardment of material results in radiation damage to the near-surface target structure and introduction of foreign atoms and electric charge to the target.[1] Energetic heavy-ion beams have been used to bombard biological materials for genetic modification purposes, particularly for the mutagenesis of living organisms including both plants and bacteria.[2–5] In this application, the deoxyribose nucleic acid (DNA) structure inside the cell nucleus is modified randomly by ion-beam irradiation and positive mutations are subsequently selected for propagation. More recently, ion-beam bombardment has been used for the direct transfer of exogenous macromolecules such as vital dye and DNA into plant cells. *GUS* gene transfer into rice cell suspension[6] and tobacco pollen,[7] and transfer of Trypan blue (a vital dye) into *Curcuma* embryo[8] induced by heavy-ion-beam bombardment have been described. In ion-beam-induced DNA transfer only the cell envelope is bombarded so as to allow a subsequent orderly transfer of whole DNA into the internal cell region. We point out that the cell envelope can be a complex structure consisting of a number of distinguishable regions such as outer membrane, cell wall, and plasma membrane,[9] and the ion bombardment must provide a path for the external DNA or other macromolecules through the thickness of the entire envelope. Although ion-beam-induced DNA transfer into plant cells, and ion-beam-induced mutation of the *LacZ* gene in the bacteria *Escherichia coli (E. coli)* (Ref. 10) have been demonstrated, ion-beam-induced DNA transfer into bacterial cells has not been reported up to now. Here, we describe our work showing that ion-beam bombardment can lead to direct DNA transfer into living bacterial (*E. coli*) cells.

To prepare the *E. coli* strain DH5α for ion bombardment, the bacteria were smeared as a thin layer on sterile adhesive tape in a petri dish that was then placed inside a sample holder. The holder was capable of sequentially exposing a number of samples to the ion beam, as well as housing an unbombarded control sample. Ion bombardment was carried out using the mass-analyzed heavy-ion-implantation facility at Chiang Mai University.[11] Argon or nitrogen ions at 26 keV bombarded the *E. coli* to fluences of 5×10^{14}, 1×10^{15}, 2×10^{15}, and 4×10^{15} ions/cm^2. Inside the target chamber the operating pressure was about 10^{-3} Pa and the temperature of the target was about 0 °C. The samples were maintained under these conditions for about 1.5–2 h, allowing for system pump-down and ion bombardment.

Our procedure was to carry out the ion bombardment as described above, followed by DNA transfer by incubating a mixture of the DNA and the ion-bombarded bacteria, followed finally by biological testing procedures to confirm that the DNA was indeed transferred. Testing for DNA transfer was done sequentially by two independent methods: detection of marker genes, and measurement of the transferred DNA molecular size. In our work, three marker genes were selected: *amp*$^+$, *LacZ*, and *GFP* genes. The *amp*$^+$ gene product is resistant to ampicillin. The *LacZ* gene product is an enzyme called β-galactosidase, which can hydrolyze an artificial chemical called X-gal to release a blue pigment.[12] The *GFP* gene product is a protein called green fluorescent protein, which can be visualized in green under ultraviolet

[a)]Electronic mail: thirapat@fnrf.science.cmu.ac.th

TABLE I. DNA plasmids used in the study.

DNA plasmid	Molecular size (kb)	Marker gene carried
pGEM2	2.8	amp^+
pGEM-T easy	3.0	amp^+, *LacZ*
pGFP	3.3	amp^+, *GFP*

(UV) light. The gel electrophoresis technique is used to measure the molecular size.[13]

We used three different DNA plasmids—pGEM2, pGEM-T easy, and pGFP—with different molecular sizes and carrying different marker genes, as shown in Table I. The ion-bombarded bacterial cell samples were removed from the vacuum chamber and promptly, within about 10–15 min, suspended in 150 μl LB^- medium for 2–5 min (LB medium, ''Luria broth,'' is a bacterial culture medium, and LB^- is LB medium without ampicillin supplement). 10–15 μl of the cell suspension were incubated separately with 10 μg of each plasmid on ice. The incubation period was 30 min and a 15 min period was also used for the pGFP case. Then, the mixtures were cultured in 3 ml LB^- at 37 °C for 2 h with vigorous shaking. For testing the property of ampicillin resistance, the cultured mixtures were added with ampicillin antibiotic to a final concentration of 100 μg/ml (the medium called LB^+) and cultured further overnight under the same conditions as above. Vacuum-treated but nonbombarded control samples were cultured in both LB^- and LB^+ media. Since pGEM-T easy and pGFP plasmids contain not only the amp^+ gene but also others, the properties of *LacZ* and *GFP* genes were subsequently tested for in the *E. coli* that had positively shown the presence of the amp^+ gene. For detecting the *LacZ* gene, the *E. coli* samples bombarded at a fluence of 2×10^{15} ions/cm^2, incubated with pGEM-T easy and cultured in LB^+, were then cultured overnight on agar-solidified LB^+ together with X-gal and a chemical inducer IPTG (for activating the gene). For detecting the existence of the *GFP* gene, the *E. coli* incubated with pGFP was further cultured overnight on solid LB^+ at 30 °C and then viewed under UV light. For the molecular-size measurements, following conventional preparation,[14] the transferred plasmids were individually digested by various restriction enzymes (*Pst*1, *Eco*R1, and *Xba*1) and then analyzed by gel electrophoresis. All the experiments were repeated at least twice.

The observed results were as follows. The control samples that were exposed to the vacuum and low-temperature environment but not ion bombarded grew well in LB^- but not in LB^+, thus indicating that the harsh conditions did not suppress cell viability. The *E. coli* bombarded with fluences of 5×10^{14}, 1×10^{15}, and 2×10^{15} ions/cm^2 grew well in LB^- but not in LB^+, whereas those bombarded with 4×10^{15} ions/cm^2 did not grow in the media, implying that the experiment on the lower-fluence ion-bombarded bacteria was meaningful. Growth of the *E. coli* into which pGEM2 was transferred was found to be dependent on the ion species and bombardment fluence. For Ar-ion bombardment with fluences of 1×10^{15} and 2×10^{15} ions/cm^2, the bacteria grew successfully in the LB^+ medium, whereas survival growth of the bacteria bombarded with a fluence of 5×10^{14} was not observed. None of the cells bombarded with any fluence of N ions and subsequently incubated with the plasmids was able to grow in the LB^+ medium, nor the control sample. The *E. coli* containing the other two transferred plasmids behaved similarly, in particular showing good growth for the bacteria treated with an Ar fluence of 2×10^{15} ions/cm^2 even though incubated for different time periods. Blue colonies were exhibited for the *E. coli* detected with the transferred *LacZ* gene, as shown in Fig. 1(a). Green colonies were visible under UV light for the *E. coli* detected with the transferred *GFP* gene, as shown in Fig. 1(b). This behavior of the color colonies demonstrates a completely successful transfer of the corresponding genes into the bacteria without loss of genes originally carried by the DNA plasmids. Successful DNA transfer was further confirmed by the measured molecular sizes. As shown in Figs. 1(c) and 1(d), the gel electrophoresis displacement locations of the pGEM-T easy and pGFP transferred plasmids correspond to standard positions of molecular sizes 3 and 3.3 kb, respectively. These results indicate that indeed the original exogenous plasmids were transferred into the ion-bombarded *E. coli*.

Physical mechanisms that offer an explanation for ion-beam-induced macromolecule transfer into cells have been discussed previously for the case of plant cells.[3,6] Two possibilities have been considered: electroporation,[15] in which pores in the plasma membrane may be opened by ion-beam-induced electric-charge transfer across the cell envelope, and etching of the cell by ion-beam sputtering leading to perforation of the cell envelope (including outer membrane, cell wall, and plasma membrane). Electroporation is a transient effect, however, occurring promptly at the time of cell charging and of gate width small compared to a second. In the present case where the DNA transfer occurs not simultaneously with ion bombardment but instead at a time of about 10 min or so after the bombardment, electroporation would seem to be ruled out.

Collisional effects of the beam in the cell envelope can be due to the energetic primary ions and/or to the lower-energy secondary particles associated with the collision cascade. Perforation of the cell envelope can be brought about by direct sputter removal (etching) of material at relatively high collision energy and also by breaking of chemical bonds resulting in damage to the cell envelope at considerably lower energy. Molecular-bond breaking can be effected not only by energetic primary ions but also by secondary particles in the collision cascade, and in this case the number of atoms displaced can be several hundreds for each primary ion. The ion range (distance below the cell surface to the peak of the implanted ion distribution) is critical for optimal perforation of the cell envelope—cell damage should extend through the thickness of the cell envelope but should be minimal in the interior cell region (cytoplasm). Estimates of ion range contain considerable inherent uncertainty because of a lack of precise knowledge of the cell envelope composition, mass density, and physical state under our experimental conditions (low pressure and temperature within the vacuum chamber). Nevertheless, Monte Carlo and analytical calculations using the TRIM code[16,17] and the PROFILE code[18] and with our best knowledge of the cell envelope[9,19] indicate

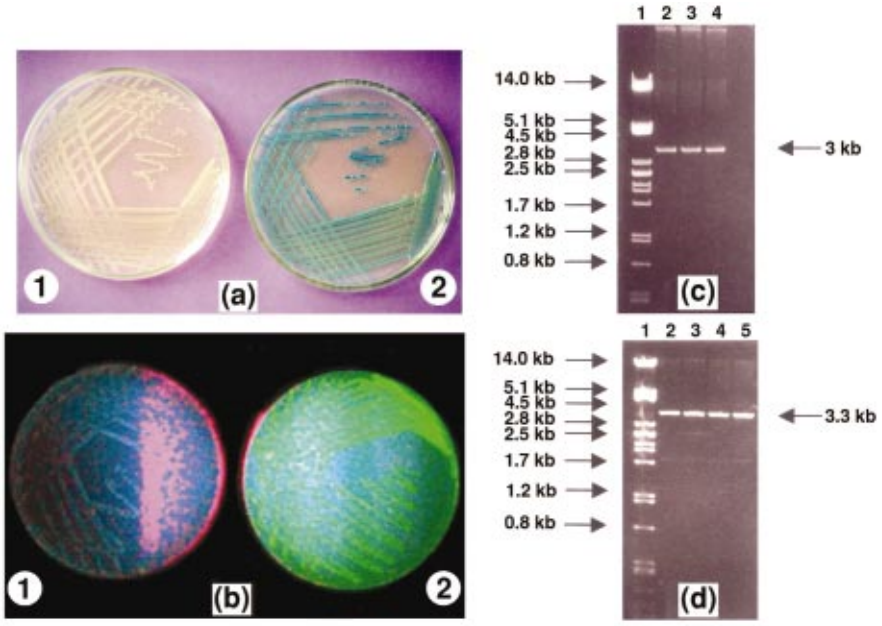

FIG. 1. (Color) Analysis of transferred pGEM-T easy and pGFP plasmids in ion-bombarded *E. coli* strain DH5α. (a) β-galactosidase activity in LB media added with X-gal and IPTG. The *E. coli* without pGEM-T easy (1), and with the transferred pGEM-T easy (2), cultured in LB^- and LB^+, respectively. (b) Expression of green fluorescent protein in *E. coli*. The *E. coli* without pGFP (1), and with the transferred pGFP (2), plated on LB^- and LB^+, respectively, visualized under UV light. (c) Measurement of molecular size of pGEM-T easy in gel electrophoresis. The transferred pGEM-T easy was digested with restriction enzymes *Pst*1 (lane 2), and *Eco*R1 (lane 3). The original pGEM-T easy digested with *Pst*1 is in lane 4. A standard molecular-size marker is shown in lane 1. (d) Measurement of molecular size of pGFP in gel electrophoresis. The transferred pGFP was digested with restriction enzymes *Pst*1 (lane 2), *Eco*R1 (lane 3), and *Xba*1 (lane 4). The original pGFP digested with *Pst*1 is in lane 5. A standard molecular-size marker is shown in lane 1.

that a range of 500–600 Å can be expected for 26 keV Ar ions, with damage due to the secondary cascade extending yet deeper. This is in good agreement with our estimates of the cell envelope thickness.[9,19] The *E. coli* cell has a 30 Å cell wall, a periplasmic space of about 150 Å, and a 75 Å plasma membrane, all surrounded by a complex outer membrane of thickness 300–700 Å as indicated by our transmission electron microscopy measurements; the entire cell envelope is thus of thickness some 500–900 Å. The range of nitrogen ions is about twice that of Ar ions of the same energy due to the smaller stopping power for the lighter ion species.[20] DNA transfer into the cells was not observed subsequent to N-ion bombardment.

The model that emerges is thus of perforation of the cell envelope by ion-beam bombardment in the appropriate regime of energy and fluence, allowing subsequent transport of the large DNA molecule into the interior cell region. When the ion range is approximately equal to the cell envelope thickness and the bombardment fluence is adequate, a pathway for macromolecule transfer through the cell envelope is formed. So long as the ion-bombardment damage is not too severe (bombardment fluence not too great) the cell envelope can recover, and the cell will live and propagate. If the ion fluence is too low, the cell envelope is not perforated and DNA transfer into the cell does not occur, and if the ion fluence is too high then the cells are damaged irreversibly. Further investigation is needed to provide a detailed explanation of the mechanisms involved in creating passages or channels through the cell envelope and enhancing the permeability through the cell envelope.

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